70803 U.S. PTO 04/30/97

A/Seq.

59383 U.S. PTO 08/846606 04/30/97

Patent Case No.: JB0600Q

ASSISTANT COMMISSIONER FOR PATENTS Washington, D.C. 20231

Sir:

Transmitted herewith for filing is the patent application including a specification and claim(s) of:

Inventor(s): RAVNIKAR *et al.*

For: EXPRESSION OF SOLUBLE HETEROLOGOUS PROTEINS IN BACTERIA UTILIZING A THIOREDOXIN/PROTEIN EXPRESSION VECTOR

Also enclosed are:

[X] 7 sheets of drawing(s).

[X] Declaration and Power of Attorney, Unexecuted

[X] A floppy disk, including the computer form of the Sequence Listing in the specification. The undersigned certifies that the computer form is equivalent in content to the Sequence Listing in the specification.

[ ] _____

For	Number Filed (1)	Number Extra (2)	Rate	Fee
Basic Fee				$770.00
Total Claims	13 – 20 =	0	0 x $22.00	$0
Independent Claims	3 – 3 =	0	0 x $80.00	$0
		TOTAL FILING FEE		**$770.00**

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[X] The Commissioner is authorized to charge Deposit Account No. 19-0365 in the amount of the above Total Filing Fee. A duplicate of this sheet is enclosed.

[X] General Authorization. This paper constitutes a general authorization to the Commissioner, for any deficiency in the above Total Filing Fee and all fee requirements subsequent to the instant filing, to charge all fees for amendments, petitions, and any and all other papers to our Deposit Account 19-0365. This is not, however, an automatic authorization to charge our Deposit Account the Issue Fee.

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JB0600Q

EXPRESSION OF SOLUBLE HETEROLOGOUS PROTEINS IN BACTERIA UTILIZING A THIOREDOXIN/PROTEIN EXPRESSION VECTOR

This application claims the benefit of U.S. Provisional Application 60/011,606, filed April 30, 1996.

BACKGROUND OF THE INVENTION

Expression of heterologous proteins in bacteria such as *E. coli* usually results in the formation of insoluble inclusion bodies that must be denatured and properly folded before the "natural" protein product is finally obtained. Thus there is a need to develop a bacterial expression system in which heterologous proteins can be expressed in the bacteria in a soluble, biologically active form.

SUMMARY OF THE INVENTION

The present invention fills this need by providing for a vector which coexpresses a heterologous protein and thioredoxin wherein the heterologous protein and the thioredoxin are expressed as separate, non-fused proteins.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows schematically the construction of plasmid pDR75.
Figure 2 shows schematically the construction of plasmid pDR85.
Figure 3 shows schematically the construction of plasmid pDR109.
Figure 4 shows schematically the construction of plasmid pDR88.
Figure 5 shows schematically the construction of plasmid pDR80.
Figure 6 shows schematically the construction of plasmid pDR102.
Figure 7 shows schematically the construction of plasmid pDR112.

DETAILED DESCRIPTION OF THE INVENTION

All references cited herein are incorporated herein by reference.

According to the process of the present invention heterologous proteins can be produced properly folded, soluble and biologically active

by the coexpression of thioredoxin and the heterologous protein in bacteria especially *Escherichia coli* (*E. coli*). However, according to the present invention, the thioredoxin and the heterologous protein must be coexpressed as separate proteins and not as fused proteins.

As used herein, the term "transformed bacteria" means bacteria that have been genetically engineered to produce a mammalian protein. Such genetic engineering usually entails the introduction of an expression vector into a bacterium. The expression vector is capable of autonomous replication and protein expression relative to genes in the bacterial genome. Construction of bacterial expression is well known in the art, provided the nucleotide sequence encoding a desired protein is known or otherwise available. For example, DeBoer in U.S. Pat. No. 4,551,433 discloses promoters for use in bacterial expression vectors; Goeddel *et al.* in U.S. Pat. No. 4,601,980 and Riggs, in U.S. Pat. No. 4,431,739 disclose the production of mammalian proteins by *E. coli* expression systems; and Riggs *supra*, Ferretti *et al. Proc. Natl. Acad. Sci.83*:599 (1986), Sproat *et al., Nucleic Acid Research 13*:2959 (1985) and Mullenbach *et al., J. Biol. Chem 261*:719 (1986) disclose how to construct synthetic genes for expression in bacteria. Many bacterial expression vectors are available commercially and through the American Type Culture Collection (ATCC), Rockville, Maryland.

In the present invention a bacterium is transformed with vector containing a gene encoding a heterologous protein and a gene encoding a thioredoxin protein. An example of such a thioredoxin gene is SEQ ID NO:3. The following examples illustrate the coexpression of thioredoxin and heterologous proteins to produce properly folded proteins. The nucleic acid or gene which encodes the thioredoxin and the nucleic acid or gene which encodes the heterologous protein should be on the same vector such as a plasmid. Furthermore, it is even more preferable that the nucleic acid or gene which encodes the thioredoxin and the nucleic acid or gene which encodes the heterologous protein should be operationally linked to a common promoter such as the *lac* promoter.

Example 1

PCR Cloning of the Thioredoxin Gene from *E. coli*

E. coli chromosomal DNA was isolated from host strain MM294 according to the BioRad Instagene procedure. PCR primers were

synthesized according to the published sequence for the thioredoxin (trxA) gene. The forward primer includes an NdeI site within the methionine start codon such that the trxA gene may be readily cloned and expressed by the cytoplasmic pMBD vectors illustrated in the figures shown below. The reverse primer includes a silent nucleotide change to generate a BsaBI site for future constructions and a BamHI site for expression vector cloning.

Forward Primer (SEQ ID NO:1)

NdeI

CCTGTGGAGT TA**CATATG**AG CGATAAAATT

Reverse Primer (SEQ ID NO:2)

BamHI BsaBI

GCACCCAACA TGCAA**GGATC** **C**TTACGCCA**G** **ATTAGCATC**G AGGAACT

This resulted in the following trxA gene (SEQ ID NO:3)

ATGAGCGATA AAATTATTCA CCTGACTGAC GACAGTTTTG ACACGGATGT
ACTCAAAGCG GACGGGGCGA TCCTCGTCGA TTTCTGGGCA GAGTGGTGCG
GTCCGTGCAA AATGATCGCC CCGATTCTGG ATGAAATCGC TGACGAATAT
CAGGGCAAAC TGACCGTTGC AAAACTGAAC ATCGATCAAA ACCCTGGCAC
TGCGCCGAAA TATGGCATCC GTGGTATCCC GACTCTGCTG CTGTTCAAAA
ACGGTGAAGT GGCGGCAACC AAAGTGGGTG CACTGTCTAA AGGTCAGTTG
AAAGAGTTCC TCGATGCTAA TCTGGCGTAA GGATCC

A PCR product of the anticipated size was obtained, NdeI/BamHI digested and cloned into NdeI/BamHI digested pMBD202020 as outlined in the figures. The insert DNA was verified to be correct by nucleotide sequence analysis and the clone was designated pDR75-11. (Figure 1)

Example 2

Construction of a regulated vector containing the trxA vector

Vector pDR75-11 is a constitutive expression vector and it was desired to have a vector in which the expression of the trxA gene could be regulated. The trxA gene from pDR75-11 was subcloned as a XbaI/BamHI fragment into pMBD112012. The resulting plasmid was

designated pDR85. The trxA gene is expressed from the lpp/lac promoter-operator and is regulated by the lacIQ repressor. (Figure 2)

Example 3

Plasmid pDR109 Construction (Figure 3)

The trx A gene was altered to include a unique XhoI restriction site to allow for easy subcloning of a downstream recombinant protein. The trxA gene was PCR amplified.

A forward primer incorporated four nucleotide changes from the wild type *E. coli* DNA sequence so as to optimize the codon usage within the first five codons because optimal codon usage has been known to increase the efficiency of translation initiation. A reverse primer includes the incorporation of the XhoI site which results in a conservative amino acid change (aspartate to glutamate) in the thioredoxin protein.

The PCR product was subcloned into pMBD112012. The resulting plasmid expresses thioredoxin as a cytoplasmic protein from the $lacI^Q$ regulated lpp-lac promoter on a pBR322 replicon.

Shown below is the resultant trxA gene in pDR109 (SEQ ID NO:4)

```
ATGAGCGATA AAATTATTCA CCTGACTGAC GACAGTTTTG ACACGGATGT
ACTCAAAGCG GACGGGGCGA TCCTCGTCGA TTTCTGGGCA GAGTGGTGCG
GTCCGTGCAA AATGATCGCC CCGATTCTGG ATGAAATCGC TGACGAATAT
CAGGGCAAAC TGACCGTTGC AAAACTGAAC ATCGATCAAA ACCCTGGCAC
TGCGCCGAAA TATGGCATCC GTGGTATCCC GACTCTGCTG CTGTTCAAAA
ACGGTGAAGT GGCGGCAACC AAAGTGGGTG CACTGTCTAA AGGTCAGTTG
AAAGAGTTCC TCGAGGCTAA TCTGGCGTAA GGATCC
```

Coexpression of thioredoxin and the recombinant protein is achieved by mimicking the translational coupling which occurs naturally in the tryptophan operon of *E. coli*. The ribosome binding site for the downstream gene is located within the 3' end of the preceding coding region and the stop and start codons of the adjacent genes are either overlapping or are immediately adjacent to each other.

The translationally coupled recombinant gene is generated by PCR amplification with a forward primer which includes the XhoI cloning site, sequences for the ribosomes binding site within the 3' end

of the trxA gene, the stop codon for trxA (TAA) and the ATG start codon and the beginning DNA nucleotides of the recombinant gene. The incorporation of the ribosome binding site sequences within the 3' end of the trxA gene results in non-conservative amino acid changes within the protein.

Example 4

Construction of a trxA/recombinant Human Interleukin-13

Vector pDR88 contains the trxA/recombinant human IL-13 (rhuIL-13) gene fusion with a gly/ser hinge linker + enterokinase cleavage site as described by LaVallie, *et al.* (Figure 4)

Linkers were attached to a rhuIL-13 clone (pLET3) which generated pDR80. The linkers contain the BsaBI site + gly/ser hinge linker + enterokinase cleavage site + rhu IL-13 codons + SstI site.

The BsaBI/BAMHI fragment from pDR80 was cloned into pDR85 to generate pDR88. (Figure 5)

Sequence of the U411/U412 linker region (SEQ ID NO:5)

BsaBI

```
GAT AAT ATT CTG GCT GGT TCT GGT TCT GGT GAT GAC GAT GAC AAG
Asp Asn Asn Leu Ala Gly Ser Gly Ser Gly Asp Asp Asp Asp Lys
---trxA-----------||Gly/Ser hinge ----||enterokinase cleavage

                                        SstI
GGT CCT GTT CCG CCG TCT ACC GCT CTG CGT GAG CTC
Gly Pro Val Pro Pro Ser Thr Ala Leu Arg Glu Leu
```

Example 5

trx Gene Translationally coupled to the rhuIL-13 Gene

A BsaBI/Sst linker was synthesized to include a ribosome binding site and coupled stop/start codon for trxA/rhu IL-13. The double stranded oligo was cloned into pDR88 to generate pDR102. (Figure 6)

Translational Coupling Sequence in pDR102 (SEQ ID NO:6)

R.B.S. IL-13 Sst I

GAAGGAGGCT GATTAA**ATGGGTCCGGTTCCGCCGTCTACCGCTCTGGAGCTC**

Recombinant Human IL-13 (rhu IL-13) was translationally coupled to thioredoxin with the following sequence: (SEQ ID NO:7)

```
    RBS    |----8bp---|
------AAG GAG GCT GAT TAA ATG----------
--trxA-----------------|Met ---rhuIL-13
```

The resultant plasmid (designated pDR102) (Figure 6) was transformed into *E. coli* host strain MM294 and fermentation analysis was done to confirm protein expression. The culture was induced for expression and grown at 15°C to maximize the accumulation of rhuIL-13 soluble protein. Cells were harvested at 48 and 68 hrs post induction. Accumulation of soluble protein immunoreactive to anti-IL-13 antibody and of monomeric (non-fused) size was observed at both times.

Example 6

Fermentation Analysis of rhu IL-10 and rhuIL-13 Production from trxA Plasmids

Alternative coupling sequences were analyzed for rhuIL-13 clones. The two alternative sequences in pDR113 and pDR114 differ from pDR102 in that the stop codon (TAA) for trxA and the start codon (ATG) for rhIL-13 overlap each other as the TAATG sequence. In addition, the spacing between the ribosome binding site (RBS) and the ATG start codon is shorter, reduced to 7 bp in pDR113 and to 4 bp in pDR114.

```
   _____RBS__|--7bp--|
-----AAG GAG GCT GAT TAATG----  (SEQ ID NO:8) pDR113
--trxA-----------     |Met--rhuIL-13
      ___RBS____|-4 bp-|
------ AAG GAG GTT TAATG---
------trxA-----------|Met---rhu IL-13 (SEQ ID NO:9)    pDR114
```

Fermentations were done at 15°C. Soluble protein is produced in pDR113 and pDR114.

Attempts were made to enhance protein expression from pDR102 by using the Tac promoter instead of the lpp-lac promoter and by

increasing plasmid copy number by utilizing the pUC origin of replication.

Plasmid pDR111 contains the pDR102 coupling expressed from the Tac promoter. Plasmid pDR112 utilizes the pDR102 coupling expressed from the Tac promoter and pUC origin of replication. (Figure 7)

Fermentations were done at 15°C. Soluble protein was produced in both pDR111 and pDR112.

Example 7

Coexpression of Thioredoxin and Recombinant Human Interleukin-10

A trxA/rhuIL-10 fusion plasmid was made and designated pDR130. Fermentations were performed at 15°C, 25°C and 37°C. Production of soluble trxA-rhuIL-10 fusion protein was greatest at 15°C and still detectable at 37°C. Protein material remained in the soluble fraction after 90 minutes centrifugation at 40,000 rpm.

Many modifications and variations of this invention can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. The specific embodiments described herein are offered by way of example only, and the invention is to be limited only by the terms of the appended claims, along with the full scope of equivalents to which such claims are entitled.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Schering Corporation

(ii) TITLE OF INVENTION: Thioredoxin/Heterologous Protein Bacterial Expression System

(iii) NUMBER OF SEQUENCES: 9

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Schering-Plough Corporation
(B) STREET: 2000 Galloping Hill Road
(C) CITY: Kenilworth
(D) STATE: New Jersey
(E) COUNTRY: U.S.A.
(F) ZIP : 07033-0530

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: diskette
(B) COMPUTER: Apple Macintosh
(C) OPERATING SYSTEM: Macintosh 7.5.3
(D) SOFTWARE: Microsoft Word 5.1a

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(vii) PRIOR APPLICATION DATA
(A) APPLICATION NUMBER: US 60/011,606
(B) FILING DATE: 30-APR-1996

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Foulke, Cynthia L.
(B) REGISTRATION NUMBER: 32,364
(C) REFERENCE DOCKET NUMBER: JB0600Q

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCTGTGGAGT TACATATGAG CGATAAAATT 30

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GCACCCAACA TGCAAGGATC CTTACGCCAG ATTAGCATCG AGGAACT 47

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 336 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
ATGAGCGATA AAATTATTCA CCTGACTGAC GACAGTTTTG ACACGGATGT 50
ACTCAAAGCG GACGGGGCGA TCCTCGTCGA TTTCTGGGCA GAGTGGTGCG 100
GTCCGTGCAA AATGATCGCC CCGATTCTGG ATGAAATCGC TGACGAATAT 150
CAGGGCAAAC TGACCGTTGC AAAACTGAAC ATCGATCAAA ACCCTGGCAC 200
TGCGCCGAAA TATGGCATCC GTGGTATCCC GACTCTGCTG CTGTTCAAAA 250
ACGGTGAAGT GGCGGCAACC AAAGTGGGTG CACTGTCTAA AGGTCAGTTG 300
AAAGAGTTCC TCGATGCTAA TCTGGCGTAA GGATCC                 336
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 336 bases pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
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CAGGGCAAAC TGACCGTTGC AAAACTGAAC ATCGATCAAA ACCCTGGCAC 200
TGCGCCGAAA TATGGCATCC GTGGTATCCC GACTCTGCTG CTGTTCAAAA 250
ACGGTGAAGT GGCGGCAACC AAAGTGGGTG CACTGTCTAA AGGTCAGTTG 300
AAAGAGTTCC TCGAGGCTAA TCTGGCGTAA GGATCC                 336
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 81 bases pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
GAT AAT ATT CTG GCT GGT TCT GGT TCT GGT GAT GAC GAT GAC AAG 45
Asp Asn Asn Leu Ala Gly Ser Gly Ser Gly Asp Asp Asp Asp Lys
 1               5                  10                  15

GGT CCT GTT CCG CCG TCT ACC GCT CTG CGT GAG CTC             81
Gly Pro Val Pro Pro Ser Thr Ala Leu Arg Glu Leu
                20                  25
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 52 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
GAAGGAGGCT GATTAAATGG GTCCGGTTCC GCCGTCTACC GCTCTGGAGC 50
TC                                                      52
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
AAGGAGGCTG ATTAAATG  18
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AAGGAGGCTG ATTAATG 17

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION SEQ ID NO:9:

AAGGAGGTTT AATG 14

WHAT IS CLAIMED IS:

1. A method for expressing a soluble heterologous protein in bacteria comprising:

transforming a bacterium with a vector wherein the vector contains a nucleic acid sequence capable of expressing thioredoxin and a nucleic acid sequence capable of expressing a heterologous protein; and

culturing the bacterium under conditions wherein the thioredoxin and the heterologous protein are expressed as separate non-fused proteins and the heterologous protein is expressed in a soluble, biologically active form.

2. The method of claim 1 wherein the vector is a plasmid.

3. The method of claim 1 wherein the nucleic acid sequence which expresses thioredoxin and the nucleic acid sequence which expresses the heterologous protein are operationally linked to a common promoter.

4. The method of claim 3 wherein the promoter is a *lac* promoter.

5. A vector which contains a nucleic acid sequence which encodes a thioredoxin protein and a nucleic acid sequence which encodes a heterologous protein wherein the vector is capable of expressing the thioredoxin protein and the heterologous protein as separate, non-fused proteins, and wherein the heterologous protein is expressed in a soluble, biologically active form.

6. The vector of claim 5 wherein the vector is a plasmid.

7. The vector of claim 5 wherein the nucleic acid sequence which encodes the thioredoxin protein and the nucleic acid sequence which encodes the heterologous protein are operationally linked to a common promoter.

8. The vector of claim 7 wherein the promoter is a *lac* promoter.

9. A bacterium transformed with an expression vector containing a nucleic acid encoding a thioredoxin protein and a nucleic acid encoding a heterologous protein wherein the thioredoxin protein and the heterologous protein are expressed as separate proteins and wherein the heterologous protein is expressed in a soluble, biologically acitive form.

10. The bacterium of claim 9 wherein the bacterium is *Escherichia coli*.

11. The bacterium of claim 9 wherein the vector is a plasmid.

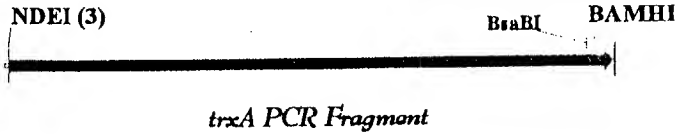
12. The bacterium of claim 9 wherein the nucleic acid which encodes the thioredoxin and the nucleic acid which encodes the heterologous protein are operationally linked to a common promoter.

13. The bacterium of claim 12 wherein the promoter is a *lac* promoter.

ABSTRACT OF THE DISCLOSURE

A method for producing a soluble, properly folded, biologically active protein in bacteria in which the heterologous protein is coexpressed with a thioredoxin protein.

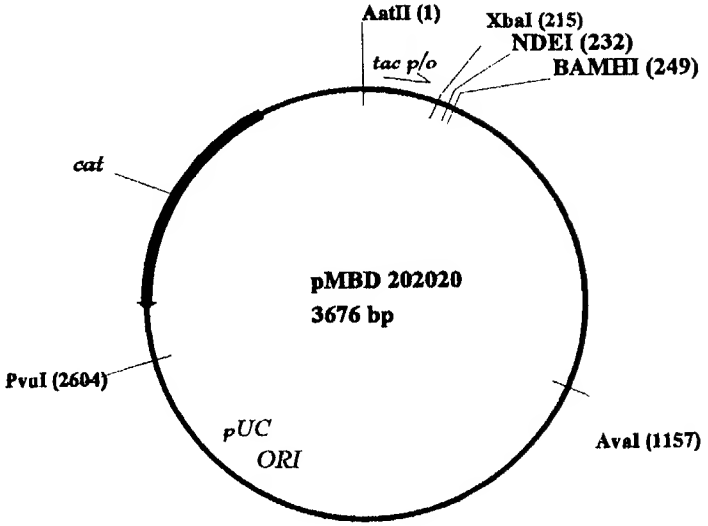
Second Figure 6



DIGEST
PCR FRAGMENT
AND
pMBD202020

NdeI/BamHI

LIGATE



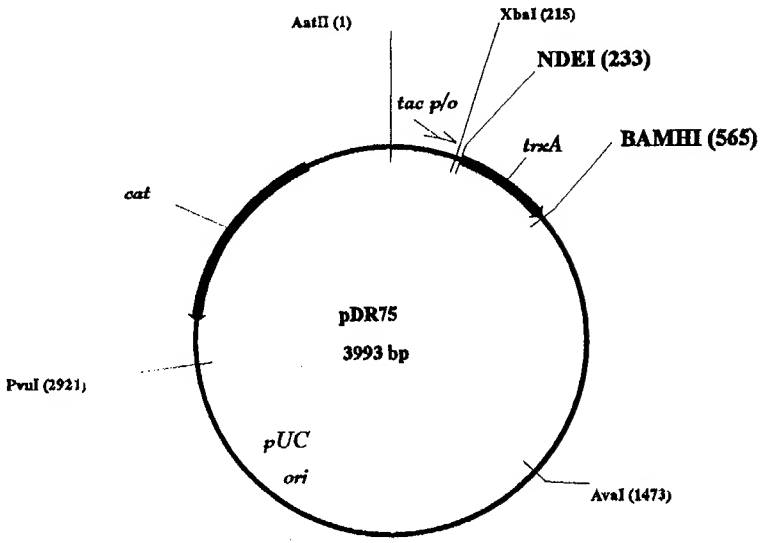
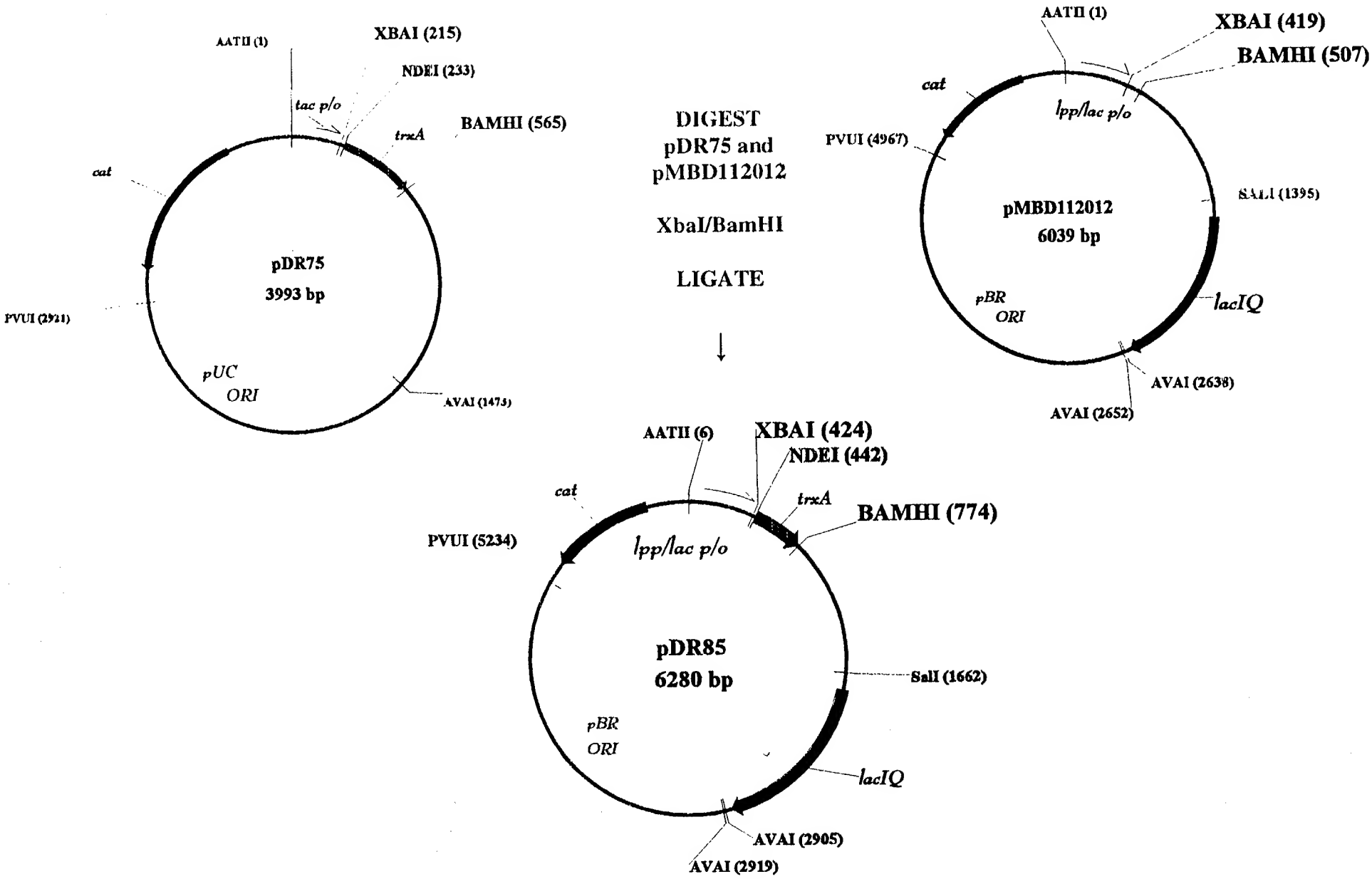


Figure 2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NDEI (233)
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trxA
BAMHI (565)
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PVUI (2921)
pUC
ORI
AVAI (1473)
DIGEST
pDR75 and
pMBD112012
XbaI/BamHI
LIGATE
AATII (1)
XBAI (419)
BAMHI (507)
cat
lpp/lac p/o
PVUI (4967)
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SALI (1395)
pBR
ORI
lacIQ
AVAI (2638)
AVAI (2652)
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XBAI (424)
NDEI (442)
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trxA
BAMHI (774)
PVUI (5234)
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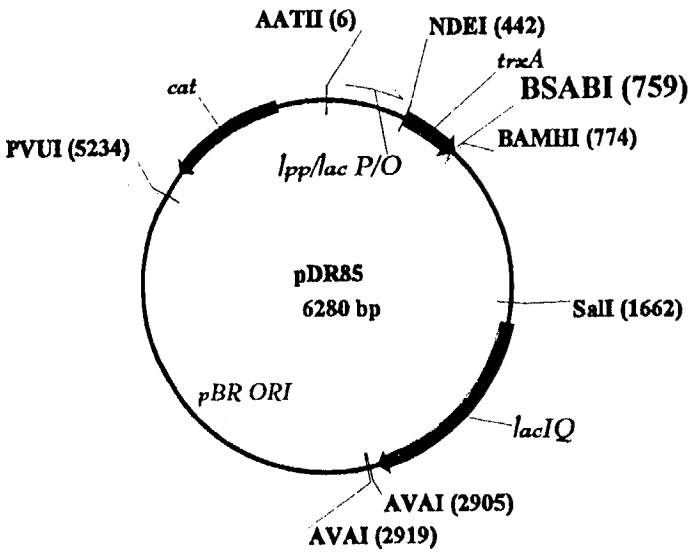
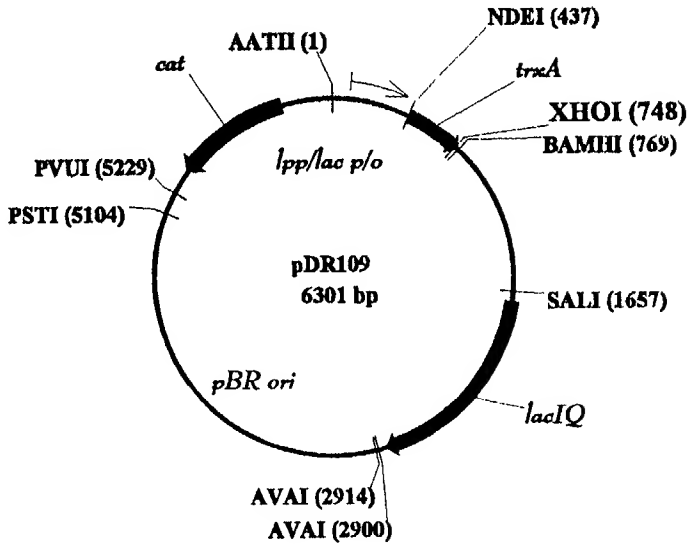
ORI
lacIQ
AVAI (2905)
AVAI (2919)

Zadeh 50 Figure 3

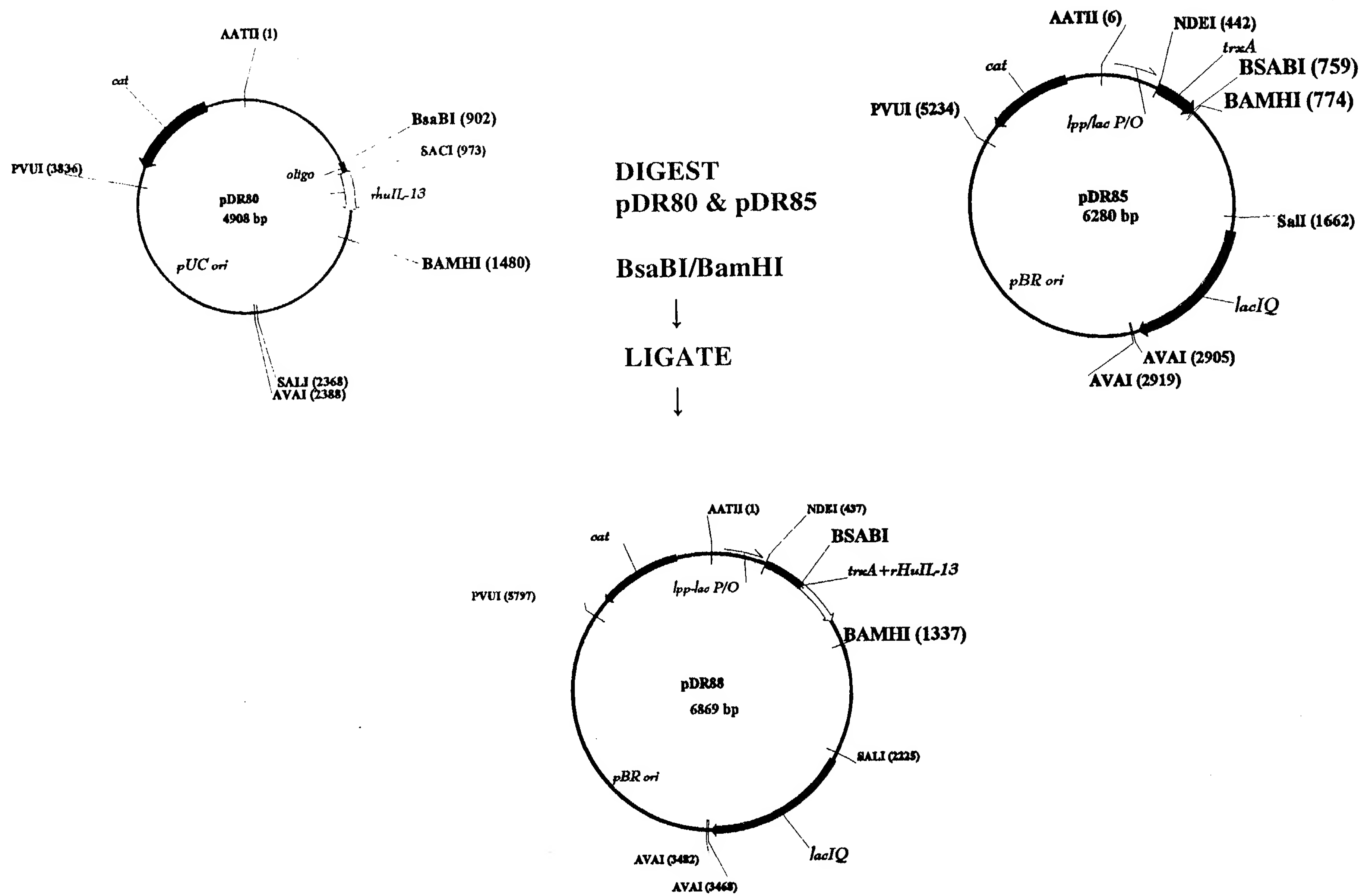




IN THE CONSTRUCTION OF pDR109 THE trxA GENE WAS ALTERED TO REPLACE THE BSABI SITE WITH A UNIQUE XHOI RESTRICTION SITE FOR EASY SUBCLONING OF A DOWNSTREAM RECOMBINANT GENE.

```
Leu Asp Ala Asn Leu Ala ***          pDR85  Sequence
CTC GAT GCT AAT CTG GCG TAA
|||  ||⇓ |||  |||  |||  |||  |||
CTC GAG GCT AAT CTG GCG TAA          pDR109 Sequence
Leu Glu Ala Asn Leu Ala ***
```

Figure 4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BsaBI (902)
SACI (973)
oligo
rhuIL-13
PVUI (3836)
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4908 bp
pUC ori
BAMHI (1480)
SALI (2368)
AVAI (2388)
DIGEST
pDR80 & pDR85
BsaBI/BamHI
LIGATE
AATII (6)
NDEI (442)
trxA
BSABI (759)
BAMHI (774)
cat
PVUI (5234)
lpp/lac P/O
pDR85
6280 bp
SalI (1662)
pBR ori
lacIQ
AVAI (2905)
AVAI (2919)
AATII (1)
NDEI (437)
BSABI
cat
trxA+rHuIL-13
PVUI (5797)
lpp-lac P/O
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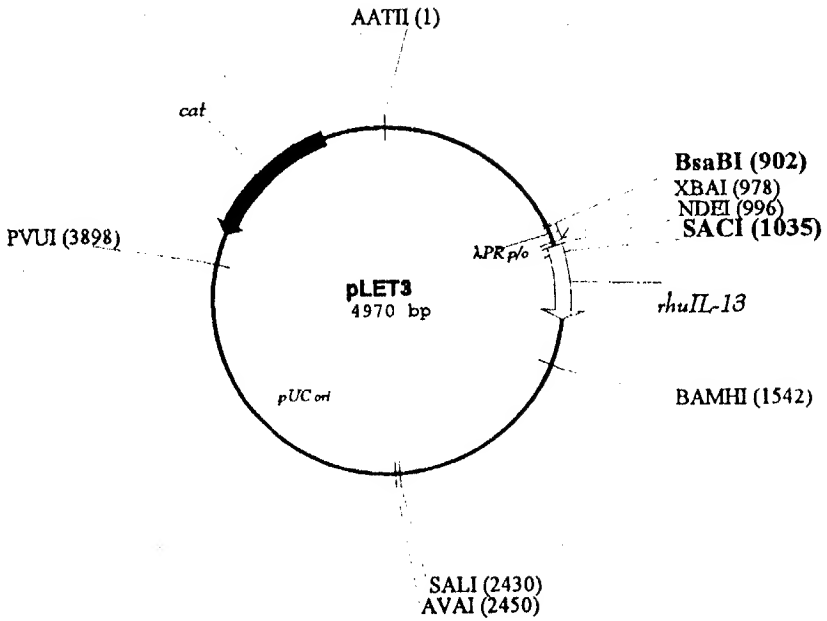
pDR88
6869 bp
SALI (2225)
pBR ori
lacIQ
AVAI (3482)
AVAI (3468)

Figure 5



BsaBI-SacI oligo sequence

GATAATAATCTGGCT	**GGTTCTGGTTCTGGT**	**GATGACGATGACAAG**
trxA sequence	*glyserglysergly linker region*	*aspaspaspasplys enterokinase cleavage*

GGTCC TGTTCCGCCG TCCGCTCTGC GTGAGCTC
rhuIL-13 sequence

Synthesize both DNA strands of the BsaBI-SacI oligo
Digest pLET3 with BsaBI-SacI

↓

Ligate pLET3 and the Oligo

↓

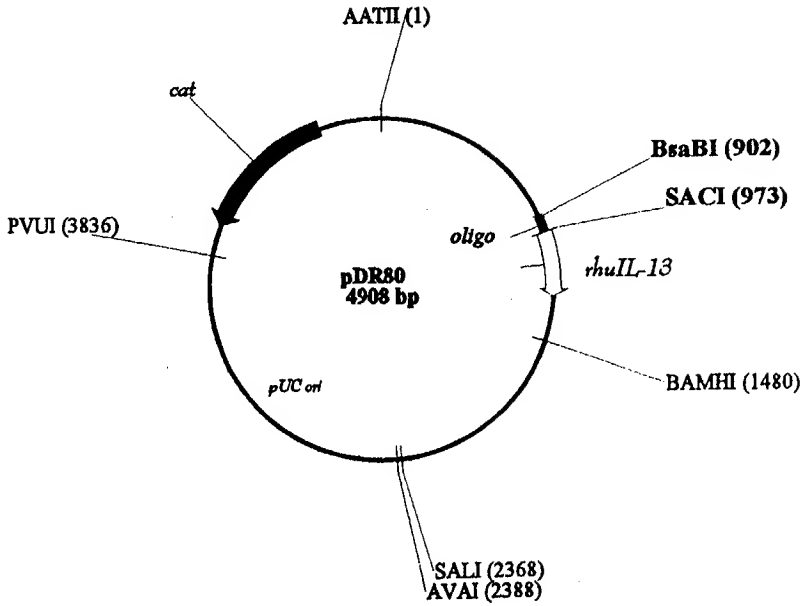
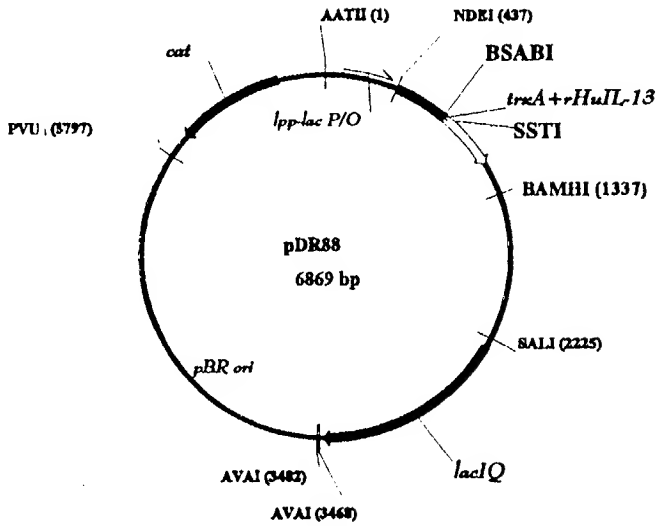
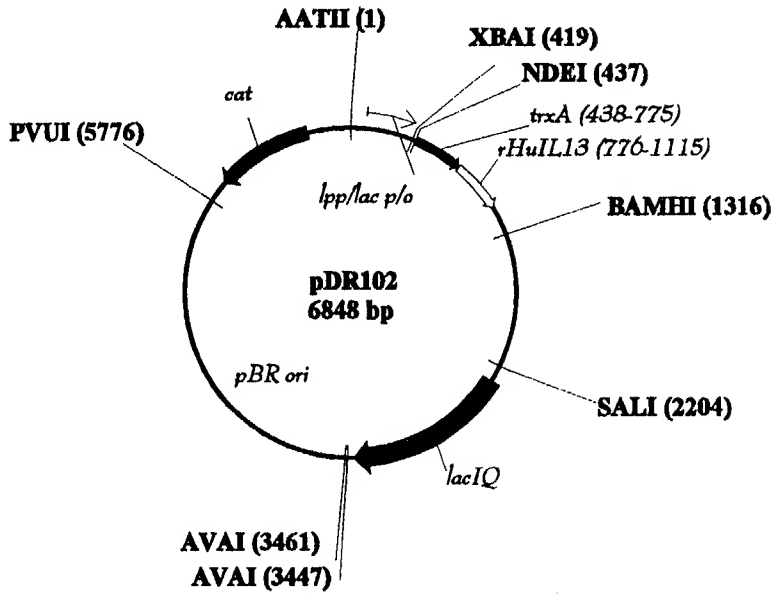


Figure 6



SYNTHESIZE A DOUBLE STRANDED OLIGO WITH BSABI/SSTI RESTRICTION SITES

LIGATE INTO BSABI/SSTI DIGESTED pDR88



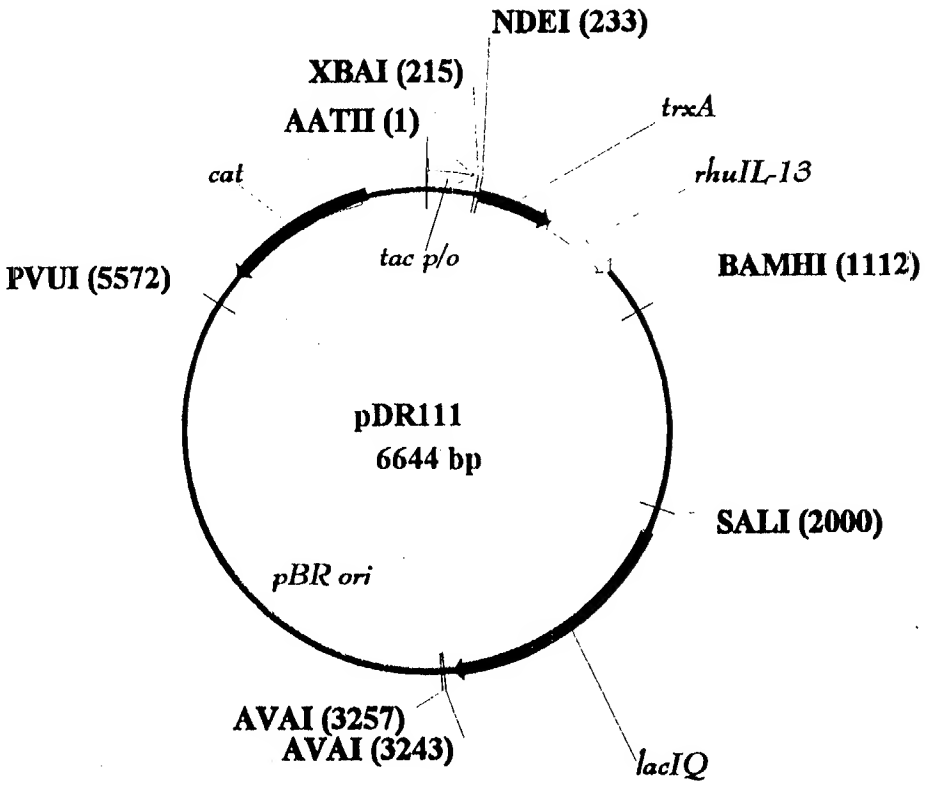
R.B.S. *rhuIL-13* SstI

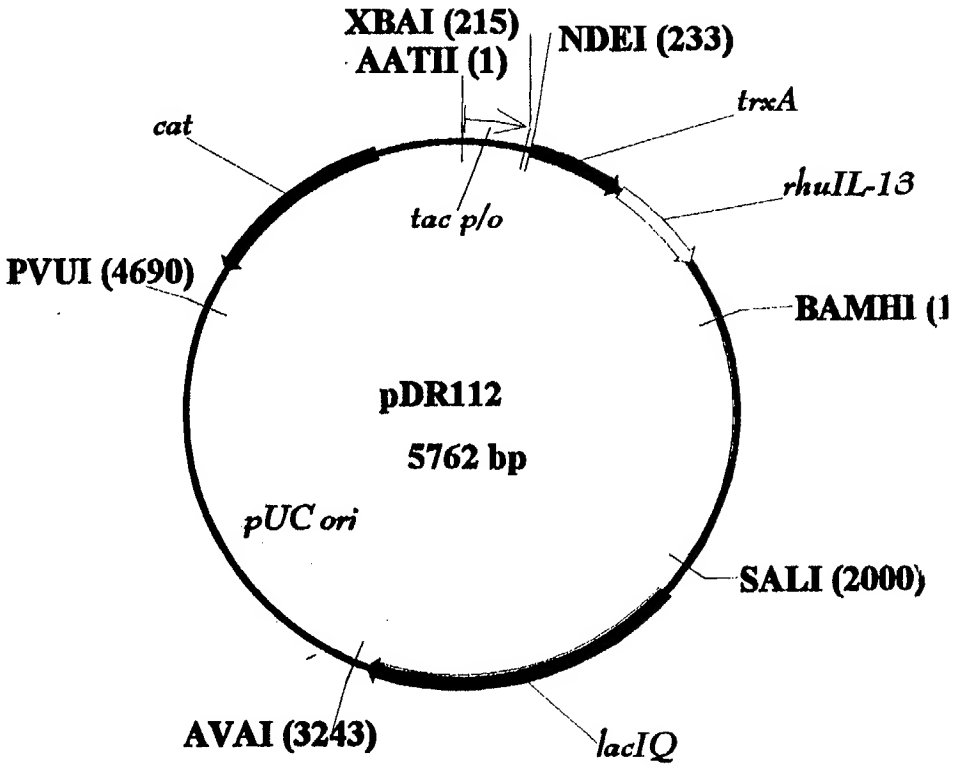
GCG AAG GAG GCT GAT TAA***ATG GGT CCG GTT CCG CCG TCT ACC GCT CTG CGT GAG CTC***

trxA

stop

Figure 7


NDEI (233)
XBAI (215)
AATII (1)
trxA
rhuIL-13
cat
tac p/o
PVUI (5572)
BAMHI (1112)
pDR111
6644 bp
SALI (2000)
pBR ori
AVAI (3257)
AVAI (3243)
lacIQ


XBAI (215)
AATII (1)
NDEI (233)
trxA
cat
rhuIL-13
tac p/o
PVUI (4690)
BAMHI (1
pDR112
5762 bp
pUC ori
SALI (2000)
AVAI (3243)
lacIQ

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

Attorney's Docket No. JB0600Q

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name;

I believe I am the original, first sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

"EXPRESSION OF SOLUBLE HETEROLOGOUS PROTEINS IN BACTERIA UTILIZING A THIOREDEXIN/PROTEIN EXPRESSION VECTOR"

the specification of which

☐ is attached hereto.

☒ was filed on April 30, 1997 as Application Serial No. 08/846,606

and was amended on _____ (if applicable).

☐ was filed on _____ as PCT International Application No. _____

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s):			Priority Claimed
(Number)	(Country)	(Day/Month/Year Filed)	Yes or No

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below:

60/011,606	April 30, 1996
(Application Number)	(Filing Date)

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.)	(Filing Date)	(Status – patented, pending, abandoned)

Power of Attorney: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in Patent and Trademark Office connected therewith. (List name and registration number.)

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I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signature of First Inventor	Signature of Second Inventor
Paula Ravnikar	Robert Greenberg
Date 9-9-97	Date 9/9/97